PCT / CA 00 / 01114

10 NOVEMBER 2000 (10.11.00)

CA 00/1114

4

REC'D 28 NOV 2000

WIPO PCT

PA 312597

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

October 16, 2000

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: ***60/156,700***
FILING DATE: ***September 30, 1999***

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

S. W. Montgomery

W. MONTGOMERY
Certifying Officer

October 16, 2000

MAX R. WOOD
1981 MCGILL COLLEGE AVENUE
SUITE 1600
MONTREAL, H3A 2Y3
QUEBEC, CANADA

This is to inform you that the document requested in your order dated 09-29-2000 is a true reproduction of the official office record copy of that document:

60156700 CERTIFIED PATENT APP AS FILED-MAIL 1

☐ The enclosed Patent Application as Filed is a reproduction of the application as originally filed and has been recorded using high quality scanning or microfilm equipment. Copies of page/papers that were not scannable have not been included, nor have pages/papers received after the original filing date. Copies of these pages/papers may be ordered separately.

☑ The enclosed document is a reproduction of the best available source of the official office record copy of that document.

If you have any questions or need additional information, please contact our Customer Service Department.

Mailing Address:
U.S. Patent and Trademark Office
Office of Public Records, Customer Service
Crystal Gateway 4, Suite 300
Washington DC 20231

Delivery Address:
U.S. Patent and Trademark Office
Office of Public Records, Customer Service
1213 Jefferson Davis Highway, Suite 300
Arlington VA 22202

For faster processing of new orders, please specify as appropriate:
Box 9 (Copy Sales) for Uncertified copies, or Box 10 for Certified copies of PTO Documents

Voice: (703) 308-9726 Fax: (703) 308-7048 E-Mail: PTCS@USPTO.GOV or Certdiv@USPTO.GOV

Ref:WM 312597

PTO/SB/16 (6-95)
Approved fo ugh 04/11/98. OMB 0651-0037
Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE

PROVISIONAL APPLICATION COVER SHEET

s a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53 (b)(2).

Docket Number	1770-232USPR FC/ntb	Type a plus sign (+) inside this box →	+

INVENTOR(s)/APPLICANT(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
CHIU	Ray	C.-J.	61 Belvedere Circle, Montreal, Quebec, Canada H3Y 1G9
SHUM-TIM	Dominique		18585 Larocque Street, Pierrefonds, Quebec, Canada H9K 1N5
GALIPEAU	Jacques		251 Morrison, Town of Mount-Royal, Quebec Canada H3R 1K7

TITLE OF THE INVENTION *(280 characters max)*

AUTOLOGOUS MARROW STEM CELL (MSC) TRANSPLANTATION FOR MYOCARDIAL REGENERATION

CORRESPONDENCE ADDRESS

France Côté

SWABEY OGILVY RENAULT
1981 McGill College Avenue, Suite 1600, Montréal

STATE	Québec	ZIP CODE	H3A 2Y3	COUNTRY	Canada

ENCLOSED APPLICATION PARTS *(check all that apply)*

X	Specification	Number of Pages	22		Small Entity Statement	
X	Drawings	Number of Sheets	3		Other (specify)	

METHOD OF PAYMENT *(check one)*

	A check or money order is enclosed to cover the Provisional filing fees		PROVISIONAL FILING FEE AMOUNT ($)
X	The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number	19-5113	$150.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[X] No

[ ] Yes, the name of the U.S. Government agency and the Government contract number are: ______________________

Respectfully submitted,

SIGNATURE ______________________ Date 09/30/1999

TYPED or PRINTED NAME France Côté

REGISTRATION NO. *(if appropriate)* 37,037

[ ] Additional inventors are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

AUTOLOGOUS MARROW STEM CELL (MSC) TRANSPLANTATION FOR MYOCARDIAL REGENERATION

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to autologous bone marrow stroma cells (MSCs) can be transplanted into the myocardium to grow new muscle fibers, which could improve cardiac function in patients with heart failure. The specific aims of the invention include: 1) using cell labeling technique to confirm the survival and differentiation of the implanted MSCs, and to identify their phenotype by both morphology and molecular markers; 2) examine the effects of the micro-environment of the implanted cells, on their differentiation and phenotype expression; and 3) the functional contribution when MSCs are implanted into an ischemic segment of the myocardium.

(b) Description of Prior Art

Heart failure is both common and deadly. In Canada, approximately 50,000 new cases of heart failure are diagnosed, and more than 300,000 patients currently suffer this condition. Heart failure is the only major cardiovascular disorder that is increasing in incidence and mortality at present, and in Class IV patients, one year mortality approaches 50% in spite of advances in drug therapy in recent years (1).

Artificial heart and mechanical cardiac assist are largely used as a bridge to transplantation today, and they still face formidable difficulties of thromboembolic complications and suitable energy source. Cardiac transplantation can have dramatic improvements in terminal heart failure patients, but its availability is severely restricted by the donor availability, as well as by the complications associated with immunosuppression. These approaches are also very expensive, straining the limited health care resources.

One exciting new approach in recent years is tissue engineering, in which various cells are cultured *in vitro* over biodegradable polymer scaffolds to create a 3-dimensional construct *in vitro*, which can then be

implanted to replace damaged tissues or organs. Upon the absorption of a biodegradable scaffold *in vivo*, these replacements tissues would not require immunosuppression if autologous donor cells were used for tissue engineering. Advances are being made in constructing cardiovascular structures, such as arteries and cardiac valves. Attempts are also being made to engineer 3-dimensional myocardial tissue blocks by seeding cardiac myocytes on a 3-dimensional scaffold, and cultured in rotating bioreactors. Without concomitant creation of a coronary vascular system within these constructs, however, such tissue engineered myocardium cannot be used therapeutically *in vivo*, as they will suffer rapid ischemic necrosis.

Such difficulties described above may be circumvented by tissue engineering neomyocardium *in vivo*. As is well known, cardiomyocyte loss from myocyte necrosis and apoptosis plays an important role in the initiation and progression of heart failure (2). "Cellular cardiomyoplasty" (3) is a potential future therapy for heart failure in which donor cells with the potential to differentiate into cardiac myocytes are implanted into the damaged myocardium in order to regenerate new muscle fibers. To date, a number of donor cells had been studied by various investigators, and they are summarized below.

A. ***Fetal Cardiomyocytes:***

Differentiated fetal cardiomyocytes retain a capacity for proliferation. Both in rodent and in canine models, fetal cardiomyocytes implanted into the myocardial wall of adult animals have been shown to be successfully engrafted, and develop into cells which are morphologically and functionally indistinguishable with the native cardiac myocytes within the recipient heart. They form gap junctions which should allow them to be depolarized and contract synchronously as a syncytium (4). By using fetal cells, however, the problem of donor cell availability becomes more formidable and the ethical issues more complex. Unlike for the use of fetal cells for treatment of neurological diseases, such as Parkinson's disease, in which engraftment of small numbers of cells may be adequate for therapeutic

effect, fetal cardiomyocyte transplantation is likely to require millions of new cells to be efficacious, and continued proliferation of engrafted myocytes cannot be expected to expand the population, once they are removed from the donor embryos.

B. Embryonic Stem Cells:

Klug et al transfected into embryonic stem cells transgene that confers resistance to a toxic drug under the control of a cardiac specific promoter. When embryoid bodies derived from these stably transfected embryonic stem cells were exposed to the toxic antibodies, only cardiac myocytes survived. These cells were harvested and injected into the myocardial wall of the adult mice, where they engrafted and formed appropriate cell-to-cell junctions, i.e. intercalated discs with desmosomes and gap junctions, with host cardiomyocytes while maintaining a morphologically differentiated state. In principle, this strategy would allow for generating large numbers of donor myocytes. The ethical issues of using embryonic stem cells are currently hotly debated.

C. Modified Adult Cardiomyocytes and Myoblast Cell Lines:

The adult cardiomyocytes are generally believed to be terminally differentiated and thus unable to proliferate. However, Li's group in Toronto had reported that adult cardiomyocytes obtained from biopsy can be induced to proliferate *in vitro*, while retaining some phenotypic characteristics of the cardiac myocytes, and they can be successfully engrafted into the myocardium. They also showed that such cells implanted into an ischemic myocardium could improve ventricular function. These interesting and potentially very important findings, however, require independent confirmation, more precise identification of cellular phenotype, and assurance against oncogenicity of the transformed cardiac myocytes before they can be considered for clinical use.

Robinson et al and other investigators implanted cells from established cell lines, such as C2 C12 cells which were originally derived from skeletal myoblasts (satellite cells). There is evidence that such cells, in

spite of their origin from skeletal muscle, may transdifferentiate in the heart acquiring certain phenotypic characteristics of a cardiac myocyte, such as the expression of Connexin-43 and the formation of desmosomes at cell junctions. Although it is very convenient to use established cell lines which can be purchased from suppliers, the concern on oncogenicity upon transplantation *in vivo*, and the need for immunosuppression may limit their application in clinical therapy.

D. Adult Skeletal Myoblasts (Satellite Cells):

Our group, in collaboration with Race Kao of East Tennessee State University, had explored the feasibility of implanting autologous myoblasts (satellite cells) harvested from the adult skeletal muscle. Using cell labeling technique and phenotype specific antibodies, we have over the last several years presented strong evidence that these myoblasts can undergo milieu-dependent transdifferentiation, and develop into striated muscle fibers with slow myosin heavy chains as well as intercalated discs expressing Connexin-43. Taylor et al confirmed that such engulfed satellite cells show ultrastructural features similar to immature cardiac myocytes, and when the implantation took place within a cryo-injured myocardium, cellular cardiomyoplasty could improve both the systolic and diastolic functions of such hearts. The advantages of using autologous skeletal myoblasts include the absence of immunological rejection, and no need to use fetal tissue with its associated ethical controversies. Nevertheless, it would require sacrificing the patient's skeletal muscle, and the concern that the number of satellite cells in the skeletal muscle, as well as the satellite cells' mitotic potential may decrease with age. The optimal "conditions" for satellite cells to transdifferentiate into cardiomyocytes have not been clearly defined, and the molecular mechanisms of milieu-dependent differentiation remain unknown.

It would be highly desirable to be provided with means to perform myocardial implantation without eliciting an immune response and without sacrificing the patient's skeletal muscle.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide with means to perform myocardial implantation without eliciting an immune response and without sacrificing the patient's skeletal muscle.

Another aim of the present invention is to provide with means using cell labeling technique to confirm the survival and differentiation of the implanted MSCs, and to identify their phenotype by both morphology and molecular markers.

Another aim of the present invention is to provide with means to examine the effects of the micro-environment of the implanted cells, on their differentiation and phenotype expression.

Another aim of the present invention is to provide with means to examine the functional contribution when MSCs are implanted into an ischemic segment of the myocardium.

In accordance with the present invention, there is provided a method of improving cardiac function in a patient with heart failure without eliciting an immune response and without sacrificing the patient's skeletal muscle; which comprises the step of transplanting autologous bone marrow stroma cells (MSCs) into said patient's myocardium to grow new muscle fibers.

The method may further comprise the step of using cell labeling technique to confirm survival and differentiation of implanted MSCs, and to identify said MSCs phenotype by both morphology and molecular markers.

The method may further comprise examining the effects of the micro-environment of implanted MSCs on their differentiation and phenotype expression.

The method may further comprise examining functional contribution of MSCs implanted into an ischemic segment of the myocardium.

The transplanting may be effected in the myocardium *in situ*, in the myocardium artery or using a catheter from within the myocardium.

The transplanting may also be effected in association with

angiogenesis factors.

Recently, preliminary studies were carried out using autologous bone marrow stroma cells (MSCs) as donor cells for myocardial implantation.

In accordance with the present invention, the rationale for using MSCs as donor cells for cellular cardiomyoplasty is as follows.

The bone marrow stroma micro-environment is a complex network of cells and extracellular matrix, which maintains the hematopoietic system throughout the life of the individual. Hematopoietic cells are found in the spaces between the marrow stroma. The marrow stroma cells (MSCs) are functionally defined as capable of supporting hematopoiesis (6), but lacking hematopoietic determinants (e.g., CD45, CD34, CD41, CD14, T- or B-cell markers and Mac-1).

Recent evidence showed that MSC definitely has a role in the "mesengenic process" for self-repair (7). The body has developed two major strategies for tissue replacement and renewal. The first way the body attempts cell repair is predicated on the remaining proliferative capacity of differentiated, functioning cells; hepatocytes and endothelial cells fall into this category. The second way is by their regeneration from residual cycling stem cells. An example in this category is the blood cells; all cells of the hematopoietic lineage are derived from a limited number of self-renewing multi-potent cells which respond to the appropriate cytokines and growth factors for differentiation.

However, it is now clear that bone marrow also contains cells that meet the criteria for stem cells of non-hematopoietic tissue, and they are currently referred to as mesenchymal stem cells, because of their ability to differentiate into cells that can roughly be defined as mesenchyma. These cells are also known as marrow stroma cells (MSCs), because they appear to arise from the supporting structures found in bone marrows.

The presence of stem cells for non-hematopoietic cells in bone marrow was first suggested by the observation of German pathologist Cohnheim 130 years ago. His work raised the possibility that bone marrow

might be the source of fibroblasts that deposit collagen fibers as part of the normal process of wound repair. Although his thesis has not yet been fully substantiated, definitive evidence that bone marrow contains cells that can differentiate into fibroblasts as well as other mesenchymal cells has been available since the pioneering work of Friedenstein (8), beginning in the mid 1970's. Friedenstein placed samples of whole bone marrow in plastic culture dishes and after about 4 hours, poured off the cells that were non-adherent to the dish, which in effect removed most of the hematopoietic stem cells and their progeny. After passage several times in culture, the adherent cells became more uniformly spindle-shaped in appearance. The striking feature of these cells was that they had the ability to differentiate into colonies that resembled small deposits of bone or cartilage. Further studies confirmed that MSCs isolated by Friedenstein's procedure were multi-potential, and readily differentiated into osteoblasts, chondroblasts, adipocytes, and myoblasts. Most importantly, it has been demonstrated that the MSCs, even after 20 to 30 cell doublings in culture were able to maintain their characteristics as multi-potential stem cells, capable of differentiating into various mesenchymal cells listed above.

In order to elucidate the biological role of the multi-potential MSCs, Pereira et al (9) carried out a highly innovative experiment. They isolated MSCs using the technique of Friedenstein et al from transgenic mice expressing a mutated collagen gene, which could be used as a cell marker by producing mutated Type I collagen molecules. These MSCs were injected intravenously into mice without this gene marker. After one week, few of the donor cells were found in the recipient mice. At one and five months, however, the donor cells accounted for 1.5 to 12% of the differentiated cells in bone, cartilage, and lung in addition to marrow and spleen. It appeared that the donor MSCs first replaced a portion of the MSCs in the bone marrow of the recipient mice. The MSCs then participated in a normal biological cycle in which MSCs in the bone marrow served as a continuing source of progenitor cells for a variety of mesenchymal tissues in the body.

Furthermore, their findings suggested that the progeny of MSCs acquired the phenotype of different target tissues, either before they left the marrow, or after they have entered the micro-environment of the tissue itself through "milieu-dependent differentiation". Caplan (7) thus postulated that MSCs participate in the "mesengenic process" which continues throughout life. This process functions to continually rejuvenate various mesenchymal tissues and ensure rapid repair of tissue injuries. Such repair involves the recapitulation of the same cellular transitional events observed during embryonic development on the scaffolding of the pre-existing macro-morphology and its signaling systems. Clinical applications of these findings have been attempted. For example, Horwitz et al reported on the initial favorable results of allogenic bone marrow transplantation in children with osteogenesis imperfecta.

The myocardium belongs to mesenchymal tissues. Can the MSCs differentiate to cardiac myocytes? Makino et al (10) recently showed that MSCs indeed could do so *in vitro* under proper conditions. After immortalizing MSCs by prolonged culture *in vitro*, they were able to identify a single clone of adherent fibroblast like cells which, when treated with 5-azacytidine, would reproducibly differentiate into adjoining myocytes with synchronous beating. Analysis of the isoforms of contractile protein genes, such as myosin heavy chain, myosin light chain, β-actin, indicated that the phenotype of these cells was similar to that of fetal ventricular cardiomyocytes. After differentiation, these cells, which they called cardiomyogenic cells, acquired many morphologic features of cardiac muscle, including sarcomeres, one to three centrally located nuclei, and atrial granules. They also expressed several cardiac specific genes, including the GATA4 and Nkx2.5 transcription factors and the brain natriuretic peptide (BNP) as well as atrial natriuretic factor (ANF) genes. They stained positive with anti-myosin, anti-desmin, and anti-actinin antibodies. In addition, they displayed cardiac like action potentials with a shallow resting membrane potential, long action potential duration, as well as a late diastolic slow

depolarization current. It should be emphasized that these findings were obtained and observed *in vitro* only, and no *in vivo* studies had been reported before the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates four days after implantation of MSC into the myocardium; Top panel: Hematoxylin and eosin stain; Lower panel:Fiuorescent microscopy picture, showing MSCs labeled with DAPI;

Fig. 2 illustrates four weeks post-implantation; and

Fig. 3 illustrates specimen four weeks after MSC implantation into the myocardium; Top panel: Hematoxylin and eosin stain; Lower panel: Fluorescent cells originated from MSCs labeled with DAPI *in vitro*.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided means to perform myocardial implantation without eliciting an immune response and without sacrificing the patient's skeletal muscle.

CLINICAL SIGNIFICANCE, POSSIBLE PROBLEMS AND FUTURE STUDIES

The ability to regenerate a functioning cardiac muscle in patients with heart failure, who have lost a significant amount of native cardiac muscle fibers through ischemic necrosis and apoptosis, should open a new approach for the therapy of heart failure. Using autologous MSCs as donor cells for such cell transplant therapy has a number of important advantages. By using autologous MSCs, we can avoid the need for fetal tissue with its ethical and legal controversies, as well as avoiding the need for immunosuppression. Unlike using modified cardiac myocytes or established cell line myoblasts, the danger of oncogenicity could be diminished. Using autologous skeletal myoblasts, although sharing many of the advantages listed above with MSCs, would however require the sacrifice of a patient's skeletal muscle, which is irreplaceable. In contrast, bone marrow puncture, a routine clinical

procedure, can be repeated to harvest MSCs for more than one occasion. The procedure is also much less invasive, and can be easily performed in patients under local anesthesia, in contrast to the excision of a major muscle mass, such as the latissimus dorsi muscle in order to harvest satellite cells. The latter procedure would likely require general anesthesia. Considering that such patients will be already suffering from severe heart failure, invasiveness of the procedure required for donor cell harvesting could be an important clinical consideration. The cell implantation procedure can be combined with other surgical operations, such as coronary bypass surgery, or by minimally invasive surgical techniques or by transvenous catheter injections.

Possible problems include false positive results associated with some cell labeling techniques as well as the interpretation of phenotype specificity associated with immunohistochemical findings. For example, a myosin-slow molecule may be detected both in Type I skeletal muscle fibers as well as in cardiac myocytes, and Connexin 43 may be expressed in immature myoblasts. Nevertheless, by employing several different cell labeling techniques and immunostain antibodies, correlating with histological and ultrastructural examinations, much of these uncertainties may be addressed.

Future studies include clinical trails and mechanistic investigations. We see minimal medical and ethical difficulties for clinical trial using this approach for a number of reasons described above. In our future study, we look forward to interacting with basic scientists to further explore the signaling mechanisms and molecules in the micro-environment responsible for MSC differentiation in the myocardium. Although such studies are outside the scope of our current three year project, we believe our findings and our technical abilities will offer a valuable basis to pursue such scientific knowledge. For example, we will be comparing differentiation of labeled cells implanted at the center, and at the periphery of the infarcts. Differentiation may be facilitated at the peripheral border zone, as reported by Atkins[29] in

a satellite cell implant study, if direct cell-to-cell contact is an important signaling mechanism for such differentiation to take place. The role of cytokines and other growth factors can be examined in the future.

PRELIMINARY STUDIES

In our preliminary study, the isogenic rats were used as donors and recipients, since as in the autotransplants, this model obviates the need for immunosuppression. Lewis rats weighting 175 to 200 grams were used in all experiments. As will be described in detail below, femoral and tibial bones were explanted from the donor rats and used as the source for bone marrow stroma cells. Details of isolation, plating and passaging techniques for MSC will also be described below. The MSCs were expanded in culture for 2 to 3 weeks and labeled with DAPI (4', 6-diamidino-2-phenylindole) before transplanting them into the lateral wall of the left ventricle of the recipient rat hearts. This was accomplished by direct injection of MSC suspension using a 28-gauge needle. At different time intervals ranging from 2 days to 4 weeks following the implantation, the hearts were harvested and studied histologically. The hearts at the implant sites were sectioned serially, and stained with hematoxylin and eosin. Slides of the adjacent section to the above were examined under fluorescent microscopy to identify DAPI, which upon binding to the DNA of the MSCs prior to implantation, can be recognized as fluorescent positive cells[25].

The sections (6 μm/thick) were also examined immunohistochemically using antibodies against myosin-slow molecules. Other serial sections from these specimens are currently undergoing studies using antibodies against the cardiac gap junctional protein, Connexin-43, and other phenotype specific antibodies, for immunolabeling and staining.

Fig. 1 illustrates a microphotograph, attached with this application, was obtained 4 days after implantation. The implant site shows a needle track created during the process of injecting the MSCs, with some inflammatory response and fibrosis within the needle track. Fluorescent

microscopic examination demonstrated the presence of the labeled MSCs implanted. Fig. 2 shows labeled cells in the injection site, immunolabeled with myosin-slow antibodies which stains red. DAPI labeled fluorescent cells within needle track made during implantation. Immunohistochemical stain with antibody against slow myosin heavy chain, which shows red color. The triangle marker points to the native cardiac myocyte, and arrows point to the appearance of myosin molecules in the cytoplasm of implanted cells.

The deep red color shows myosin-slow heavy chains in the native muscle (triangle), whereas the red stain adjacent to the labeled cells suggest the synthesis of myosin-slow molecules in the implanted cells (arrows). Fig. 3, a photograph taken a short distance away from the implant needle track, shows migrated or infiltrated MSCs appearing to have differentiated fully and were incorporated into cardiac muscle fibers, morphologically indistinguishable with the native myocardium. Clear labeling of these cells can be demonstrated under fluorescent microscopy. Morphologically, they appear identical to the native myocardial fibers. Photograph taken from the myocardium adjacent to the needle track, where the implanted cells had migrated or infiltrated.

Additional studies are proposed below to confirm these findings, as well as to further elucidate the phenotypes of the new muscle using additional specific antibodies. These preliminary studies, however, clearly demonstrated the capability of our laboratory to carry out various experimental techniques, ranging from isolation, culture and identification of MSCs, as well as implanting these cells to the rat hearts with virtually no mortality.

EXPERIMENTAL PROTOCOL:

The Rationale for Experimental Models

In clinical application of the present invention, we foresee the use of autologous MSCs for cardiac implantation, in order to avoid the need for immunosuppression. Thus, we choose to use relatively inexpensive isogenic

Lewis rats for our study. The preliminary investigation described above indicates that this is a useful and reliable model, with little operative mortality for cardiac cell implantation in our hands. Furthermore, from our previous experience, we have the capability of performing coronary artery ligation and sequential echocardiographic studies in such animals, which again strengthens the feasibility of our proposed experiments.

Experimental Design

For each rat receiving cardiac cell implant (experimental animal), there will be an isogenic rat to serve as the donor of bone marrow stroma cells. Another group of sham operated rats (controls) will undergo identical surgical procedures as the experimental animals, but will receive injection of cell culture media without MSCs.

a) *Donor rats* will be sacrificed, and their femoral and tibial bones will be used to isolate, select and culture MSCs *in vitro* for 2 weeks using the technique described below. Then the cells will be collected, labeled and injected into the myocardium of the experimental recipient rats.

b) *Cell implant recipient (experimental) rats:* The prospective future recipients for cell implant will undergo thoracotomy and ligation of the anterior descending coronary artery (see below). The chest will be closed and the animals monitored with weekly echocardiographic studies to observe changes in the ventricular wall motion of the ischemic zone for 2 weeks. In the second thoracotomy, these rats will receive injection of isogenic MSCs cultured and labeled *in vitro*. The injections will be made through a 26-gauge needle into the anterolateral wall of the left ventricle, both at the center of the infarct zone, as well as at the peripheral border zone between the infarcted and non-infarcted cardiac muscles. Following the implant procedure, the chest will be closed and studied weekly using echocardiography as described below. They will be sacrificed after cell implantation at an interval of 4 days, 2 weeks, 4 weeks, and 3 months, with a sample size of 10 rats each. Frozen sections will be made serially through the implant site, at a thickness of

approximately 6 μm each. and slides will be process for fluorescent microscopic, histological, immunohistochemical and electron microscopic studies as described in detail below.

c) *Sham operated (control) rats:* This group will undergo coronary artery ligation and cardiac implantation procedures exactly as described above for the experimental group. However, instead of receiving cultured and labeled MSCs, they will receive the same volume of culture media only. They will be harvested at the same time intervals and studied in the same manner as described for the experimental animals.

Sample Size

The sample size of 10 was based on our preliminary study as this is a highly reliable model with minimal operative mortality in our hands. We expect to find no implanted cells at all in our controls; while in the experimental animals, we expect to see them in virtually all of them. Based on our experience in the preliminary study, we think 10 rats in each group will give us clear results as to the validity of our study in proving our hypothesis, although we have no preliminary data on wall motion study to calculate the sample size required.

All animals will receive humane care and all experiments will be performed according to the "Guidelines to the Care and Use of Experimental Animals" of the Canadian Council on Animal Care.

Isolation, Plating and Passaging Techniques of Bone Marrow Stroma Cells:

Isolation and primary culture of MSCs will be performed according to Caplan's method. After overdose with pentobarbital (100 mg/kg given intraperitoneally) (MTC Pharmaceuticals, Cambridge, Ontario), the femoral and tibial bones of the donor Lewis rats (weighing 175 to 200 grams) will be collected and the adherent soft tissue removed. Meticulous dissection of the long bones will be carried out in order to remove soft tissue to ensure that myogenic precursors are not carried into the bone marrow preparation. Both

ends of the bones will be cut away from the diaphysis with bone scissors. The bone marrow plugs will be hydrostatically expelled from the bones by insertion of 18-gauge needles fastened to 10 ml syringes filled with complete medium; the needles are inserted into the distal ends of the femoral and proximal ends of the tibial bones, and the marrow plugs expelled from the opposite ends. The marrow plugs are disaggregated by sequential passage through 18-gauge, 20-gauge and 22-gauge needles and these dispersed cells are centrifuged and resuspended twice in complete medium. Cell viability is assessed by the trypan blue exclusion test. After the cells are counted in a hemocytometer, $5x10^7$ cells in 7-10 ml of complete medium are to be introduced into 60 mm polystyrene tissue culture dishes (Corning, Inc., Corning, NY), which are coated in advance with a layer of laminin (Sigma) to promote marrow stroma cell adherence. Three days later, the medium is changed and the non-adherent cells discarded. The medium is completely replaced every 3 days. In approximately 10 days after seeding, the dishes will become nearly confluent and the adherent cells can be released from the dishes with 0.25% trypsin in 1 mmol/L sodium ethylenediaminetetraacetic acid (Gibco Laboratories, Grand Island, NY), split 1:2, and seeded onto fresh plates. After these twice passaged cells become nearly confluent, they can be harvested and used for implantation experiments described below after being labeled with DAPI. The "complete medium" mentioned above for our culture consists of Dulbecco's modified Eagle's medium (DMEM, Gibco Laboratories) containing selected lots of 10% fetal calf serum (FCS; JR Scientific Inc., Woodland, CA), and antibiotics (Gibco Laboratories; penicillin G, 100 U/ml; streptomycin 100 µg/ml, amphotericin B 0.25 µg/ml) at 37°C in a humidified atmosphere of 5% CO_2.

Bone Marrow Stroma Cell Labeling

Although in our preliminary studies, we have used DAPI for cell labeling, with the expertise and experience of the co-investigator (J. Gallpeau), we will use retroviral vectors as the tool for cell labeling. Retroviral vectors permit insertion of foreign synthetic genetic information in

target cells. Reporter genes such as ß-galactosidase, can therefore be stably integrated in chromozomal DNA. Expression of these transgenes permits ambiguous identification, by classic histochemical or fluorescence microscopy, of gene modified cells. As an example, retroviral labeling of hematopoietic stem cells is routinely carried out in the study of bone marrow transplantation in rodents. Undifferentiated (gene labeled) progeny cells of a widely different phenotype (lymphocytes, granulocytes, platelets, red blood cells) are readily detectable up to one year after bone marrow transplantation. We propose that retroviral labeled stroma cells that survive, proliferate or differentiate following cardiac implantation, will preserve and express the transgene as is classically observed in animals transplanted with retrovirus labeled hematopoietic stem cells. With this technique, important questions regarding bio-distribution of transplanted stroma cells can be addressed. Unambiguous identification of "labeled stroma cells", as well as their differentiated progeny, local and distant to the site of implantation in the injured heart, will become possible. Identification of other "unexpected" progeny cells, such as endothelial cells, interstitial cells, possibly others, will also be feasible. Furthermore, very sensitive PCR-based techniques, now developed in our laboratory (J. Galipeau), can detect as little as 1 to 100,000 transgene positive cells from tissue DNA extracts.

We have also demonstrated in our laboratory that cultured primary marrow stroma cells are readily transduced with synthetic retroviral vectors. High efficiency gene transfer in cultured stroma cells is now routinely carried out in our laboratory, and genetically labeled cells can be expanded *in vitro* for up to 3 to 4 weeks without loss of reporter gene expression (in this case the green fluorescent protein). We have on hand a high titer VSV-G pseudotyped ß-gal retroviral producer. We propose that transplanted stroma cells genetically labeled with a ß-galactosidase retrovector will be readily identified by classic X-Gal staining of cardiac tissue sections. These labeling procedures and experiments will be carried out under the direct supervision of J. Galipeau, co-applicant.

Coronary Artery Ligation Model in Rats

The recipient rats, both experimental and control, are isogenic Lewis rats weighing 175 to 200 grams. Anesthesia is induced and maintained with isoflurane (MTC Pharmaceuticals). The animals are intubated with an 18-gauge intravenous catheter and connected to a Harvard rodent ventilator (Harvard Apparatus Co., Inc., South Natick, Mass.) at 85 breaths per minute. The heart is exposed via a 1.5 cm left thoracotomy incision. Under direct vision, using a 5-0 prolene suture, the anterior descending coronary artery which is visible in the epicardium is ligated proximally. The thoracotomy is closed with 4-0 monofilament sutures. The muscle and skin layers are closed with 4-0 absorbable sutures and the animals are returned to their cages with filter tops. After the learning curve, the operative mortality is virtually nil.

Cell Transplantation into the Rat Heart

The recipient rats which underwent coronary artery ligation 2 weeks previously will undergo a second operation. Anesthesia and thoracotomy will be performed in the manner described above. Under direct vision, the MSC suspension is injected into the lateral wall of the left ventricle with a 20-gauge needle, both at the center of the ischemic segment of the myocardium, and at the border zone at the junction between the infarcted and normal myocardium. The thoracotomy closure and post-operative care are similar to that described above, and the animals sacrificed at intervals after this procedure, as stated earlier.

The sham operated control rats will undergo an identical procedure as described for cell implantation in the experimental animals. The only difference is that instead of injecting cultured MSCs, an identical volume of culture media (component described above) will be injected.

Echocardiographic Studies on Wall Motion

Transthoracic Doppler echocardiographic studies will be performed

in the rats every week following implantation. The rats are anesthetized as described above, the chest wall shaved, and echocardiography performed using our echo system equipped with a 7.5 MHZ transducer (Hewlett Packard Sonos 2500). A 2-dimensional short axis view of the left ventricle is obtained at the level of the papillary muscle to record M-mode tracing. Anterior and posterior end-diastolic and end-systolic wall thickness and LV diameters are measured using the American Society of Echocardiography Lineage Method, from at least three consecutive cardiac cycles. The changes in wall thickness and ventricular segmental wall motion and diameter will be recorded on videotape, and assessed by blinded independent echocardiographers.

Morphological and Immunohistochemical Studies

The heart specimens obtained from the recipient rats at various intervals will be perfused with 100 ml of saline through the posterior wall of the left ventricle, avoiding the transplant area, then processed for frozen sections. The lateral wall of the left ventricle is isolated from the remainder of the heart. Sections 6 μm thick are cut from the hearts and successive sections collected by gelatine coated glass slides. This ensures that different stains could be applied on successive sections of the tissue cut through the transplanted area (Figs. 1 and 3). One of the sections is mounted and stained with X-Gal, to identify and view the ß-gal labeled donor cells. An adjacent section is stained with hematoxylin and eosin as described in the manufacturer's specification (Sigma Diagnostics) to depict nuclei, cytoplasm and connective tissue. Other adjacent sections will be immunolabeled using various antibodies for immunohistochemical evaluation in order to identify phenotypic expression at the molecular level. These antibodies include those against myosin-slow molecules, cardiac gap junctional protein Connexin 43, desmin, and sarcomeric myosin (MF 20). Finally, specimens will also be processed and sent for ultrastructural examination in our future studies.

OTHER ADVANTAGES

We foresee that labeled cardiac myocytes and fibers will be present in the implant site, which will exhibit positive immunohistochemical stains as one may expect in normal or immature cardiac myocytes. Echocardiographic studies may demonstrate improved systolic thickening of the ischemic ventricular wall segment, and reduced ventricular size and remodeling, as had been reported following the implantation of other donor cells.

While the invention has been described in connection with specific embodiment thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses or adaptations of the invention following in general, the principles of the invention and including such departures from the present disclosure as come within the known customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of improving cardiac function in a patient with heart failure without eliciting an immune response and without sacrificing the patient's skeletal muscle; which comprises the step of transplanting autologous bone marrow stroma cells (MSCs) into said patient's myocardium to grow new muscle fibers.

2. The method of claim 1, which further comprises the step of using cell labeling technique to confirm survival and differentiation of implanted MSCs, and to identify said MSCs phenotype by both morphology and molecular markers.

3. The method of claim 1, which further comprises examining the effects of the micro-environment of implanted MSCs on their differentiation and phenotype expression.

4. The method of claim 1, which further comprises examining functional contribution of MSCs implanted into an ischemic segment of the myocardium.

5. The method of claims 1 to 4, wherein said transplanting is effected in the myocardium *in situ*, in the myocardium artery or using a catheter from within the myocardium.

6. The method of claims 1 to 4, wherein said transplanting is effected in association with angiogenesis factors.

ABSTRACT OF THE INVENTION

The present invention relates to a method of improving cardiac function in a patient with heart failure without eliciting an immune response and without sacrificing the patient's skeletal muscle; which comprises the step of transplanting autologous bone marrow stroma cells (MSCs) into said patient's myocardium to grow new muscle fibers. The method may further comprise the step of using cell labeling technique to confirm survival and differentiation of implanted MSCs, and to identify said MSCs phenotype by both morphology and molecular markers. The method may further comprise examining the effects of the micro-environment of implanted MSCs on their differentiation and phenotype expression. The method may further comprise examining functional contribution of MSCs implanted into an ischemic segment of the myocardium.

REFERENCES:

1. L'enfant C: Fixing the failing heart. Circulation 1997;95:771-772.
2. Olivetti G, Abbi R, Quaini F, et al: Apoptosis in the failing human heart. N Engl J Med 1997;336:1131-1141.
3. Chiu RC-J, Zibaitis A, Kao RL: Cellular cardiomyoplasty: Myocardial regeneration with satellite cell implantation. Ann Thorac Surg 1995;60:12-18.
4. Soonpaa MH, Koh GY, Klug MG, et al: Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. Science 1994;264:98-101.
5. Taylor DA, Atkins BZ, Hungspreugs P, et al: Regenerating functional myocardium: Improved performance after skeletal myoblast transplantation. Nat Med 1998;4:929-933.
6. Lichtman MD: The relationship of stromal cells to hemopoietic cells in marrow. In *Long Term Bone Marrow Culture*, pp. 57-96, DG Wright, JS Greenberger (eds). Alan R. Liss, New York, 1984.
7. Caplan AI: The mesengenic process. Clinics Plast Surg 1994;21:429-435.
8. Friedenstein AJ, Gorskaja U, Kulagina NN: Exper Hematol 1976;4:276.
9. Pereira RF, Halford KW, O'Hara MD, et al: Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc Natl Acad Sci 1995;92:4857-4861.
10. Makino S. Fukuda K, Miyoshi S, et al: Cardiomyocytes can be generated from marrow stromal cells *in vitro*. J Clin Invest 1999;103:697-705.

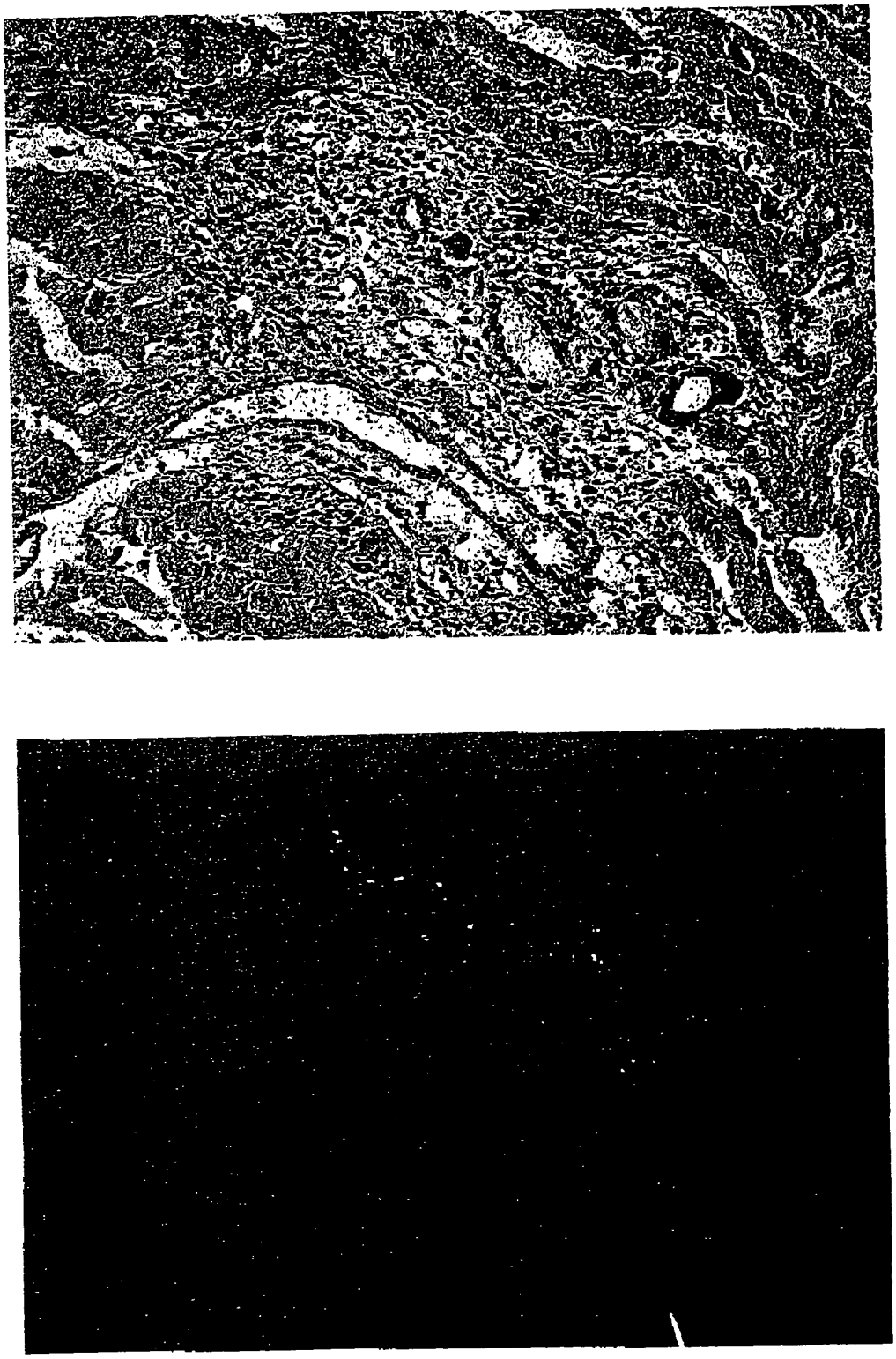
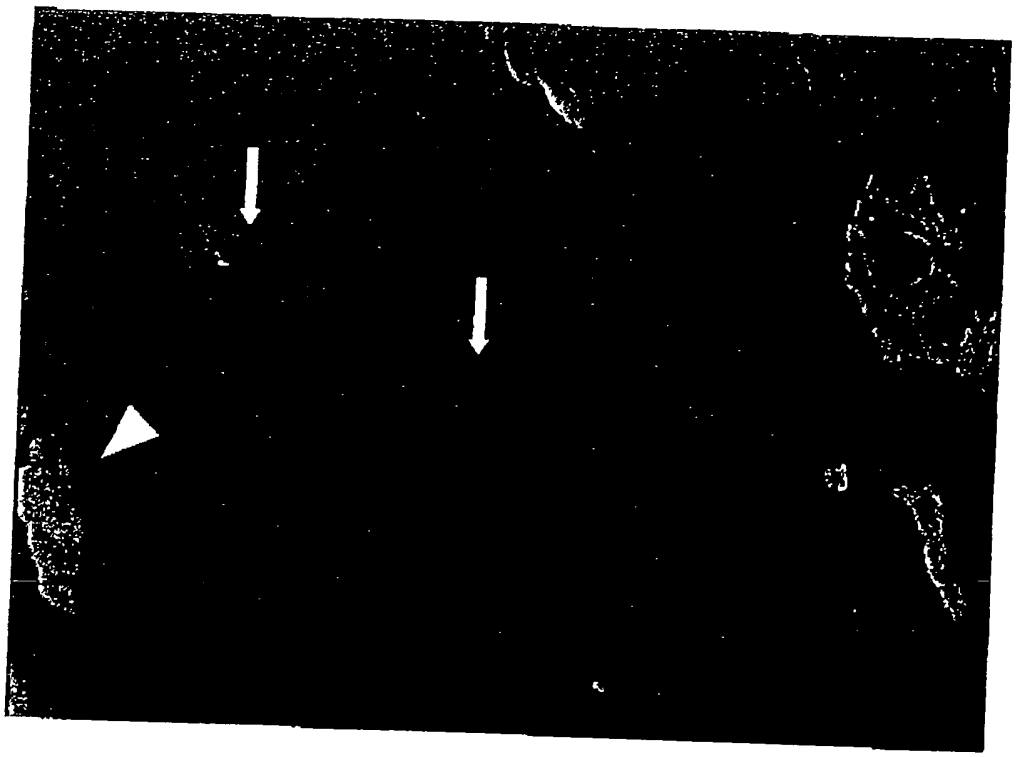


Fig. 1



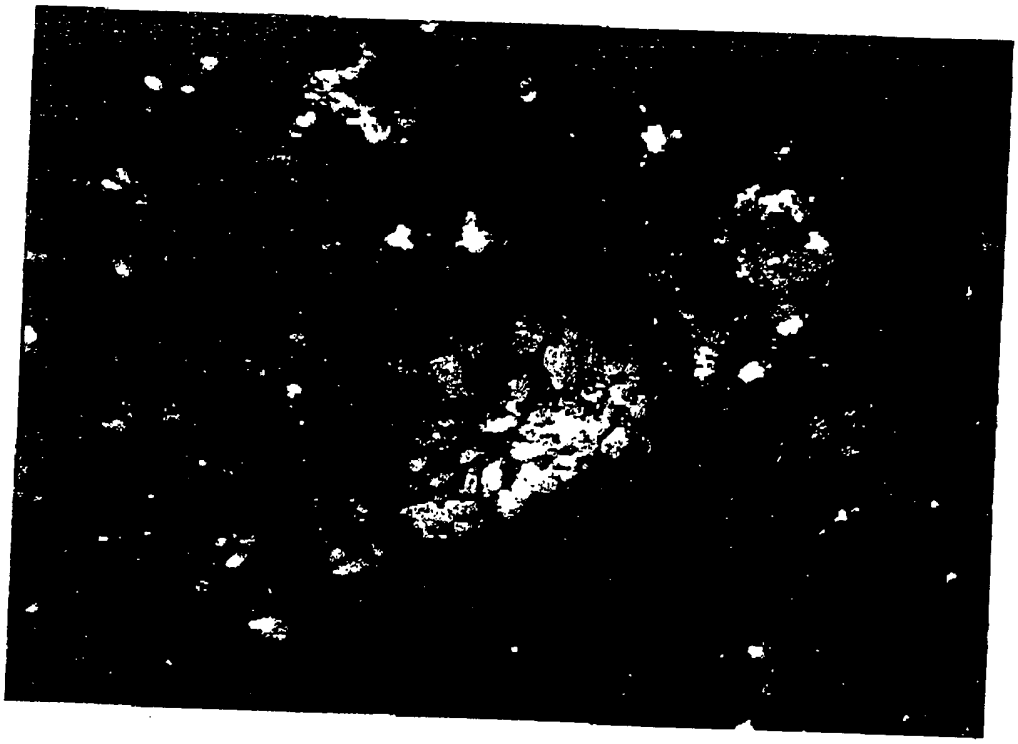


Fig. 2

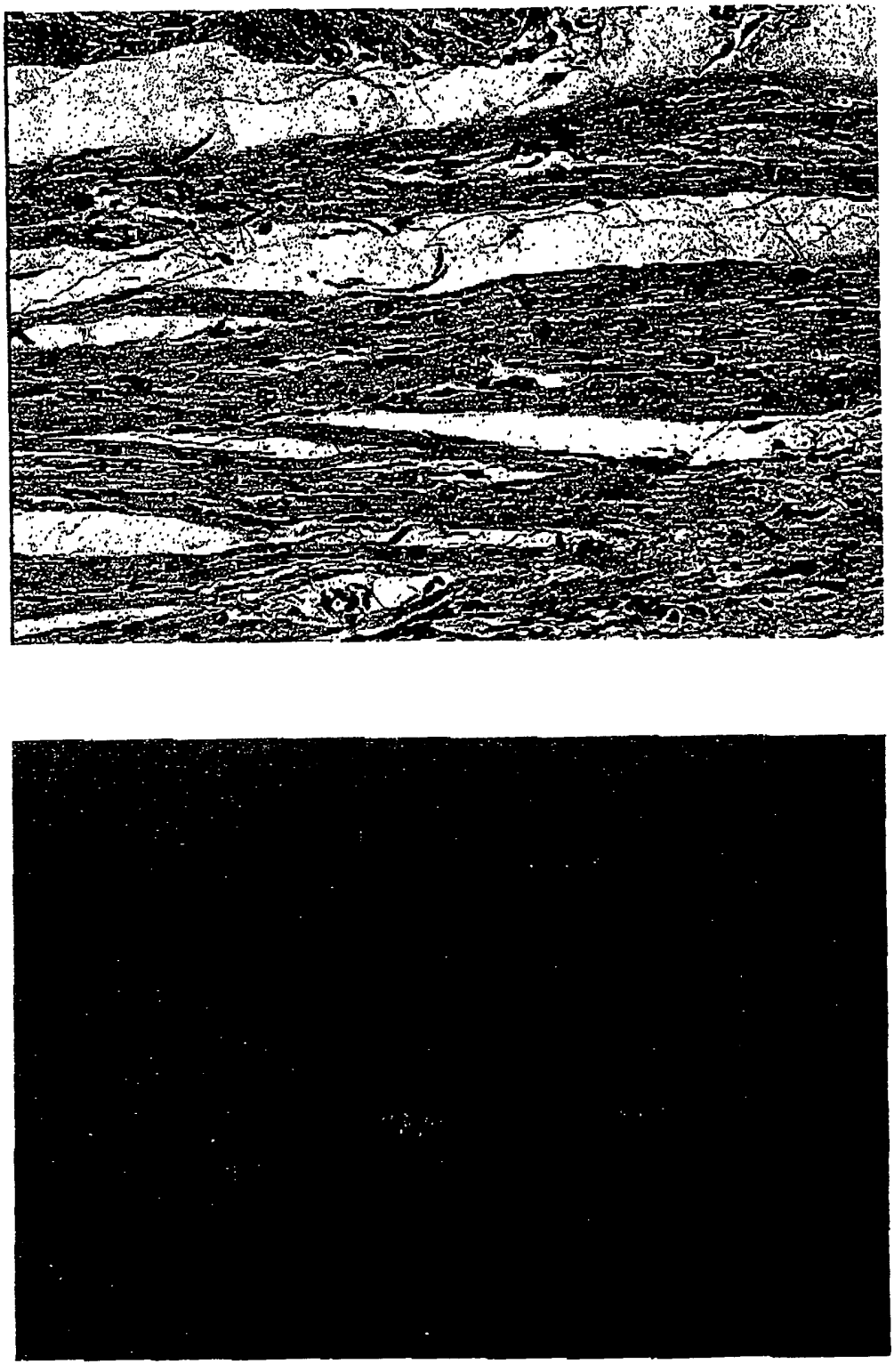


Fig. 3